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EFFECT OF D-PENICILLAMINE AND β-AMINOPROPIONITRILE ON COLLAGEN BREAKDOWN OF CARRAGEENIN GRANULOMA IN RATS

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Abstract—The effects of D-penicillamine and β-aminopropionitrile on collagen breakdown of carrageenin granuloma was studied by measuring the amounts of dialysable hydroxyproline formed during incubation *in vitro* of minced granulation tissue from rats treated with the drugs. D-Penicillamine or β-aminopropionitrile fumarate were injected into the granuloma pouch for 7 or 8 days after carrageenin injection. The collagenolytic activity of granulation tissue was increased by β-aminopropionitrile fumarate treatment, while D-penicillamine was ineffective. The changes in tissue collagenolytic activity are discussed in connection with the increased solubility of the tissue collagen.

D-Penicillamine and BAPNf* have been shown to enhance the solubility of tissue collagen and reduce its tensile strength [1–3]. The increased solubility in neutral salt solutions reflects the absence of inter- and intra-molecular cross-links [4]. Neither of the drugs affected the rate of collagen synthesis [1, 5], but their effect on the breakdown of tissue collagen is not yet well understood. In the present investigation the collagenolytic activity of granulation tissue obtained from rats treated with D-penicillamine or BAPNf was studied in connection with the solubility of the tissue collagen.

EXPERIMENTAL

Treatment of animals. A subcutaneous granuloma pouch was induced on the back of male rats of the Donryu strain weighing 110–140 g by injecting a 2% solution of Seakem 202 carrageenin (Marine Colloid Inc., Springfield, N. J., U.S.A.) according to the procedure previously described [6].

BAPNf (80 mg per rat in a volume of 0.5 ml) was injected daily into the granuloma pouch for 8 days commencing the day after carrageenin injection. Control animals were given 0.9% NaCl. Animals were sacrificed 6 hr after the last injection of BAPNf. D-Penicillamine (40 mg per rat in a volume of 0.5 ml, kindly supplied by Taisho Pharmaceutical Co., Ltd., Tokyo) with pyridoxine HCl (1 mg per rat) was injected into the granuloma pouch at 12-hr intervals from day 1 to day 6, while control animals were given 0.5 ml of 0.9% NaCl. Animals were sacrificed on day 7. Granulation tissue was harvested from the rats having a large granuloma pouch immediately after sacrifice.

Incubation in vitro *of granulation tissue.* The collagenolytic activity of granulation tissue was determined according to the procedure described in a previous report [7]. Briefly, the minced granulation tissue (800 mg) was incubated under an atmosphere of 95% O_2–5% CO_2 at 35° for 6 hr in 8 ml of Krebs' saline serum substitute [8] containing 0.8 mg each of potassium penicilline-G and dihydrostreptomycin sulfate. After incubation, the mixture was homogenized in a glass homogenizer at 1°. The homogenate was dialysed against 50 ml of distilled water at 4° for 5 days. The amount of free hydroxyproline in the dialysate was measured by the method of Kivirikko *et al.* [9]. The amount of dialysable total hydroxyproline was measured by the method of Kivirikko *et al.* [9] after hydrolysis of an aliquot of the dialysate with 6 N HCl at 105° for 16 hr. Amounts of free and dialysable total hydroxyproline formed during incubation were calculated by subtracting the values of the non-incubated samples from those of incubated samples, respectively. The amount of dialysable total hydroxyproline formed was used as an index of tissue collagenolytic activity.

Fractionation of tissue collagen. An aliquot (1.0 g) of minced granulation tissue was homogenized in 30 ml of ice-cold 1 M NaCl with a Vir-Tis homogenizer. The homogenate was shaken for 2 days at 4° and then centrifuged at 8000 *g* for 20 min at 1°. The precipitate was suspended in 30 ml of 1 M NaCl and the extraction was repeated three times. The supernatants were pooled and filtered. The filtrate was referred to as neutral salt-soluble collagen (NSC). Insoluble collagen (IC) was obtained as gelatin by autoclaving the residue remaining after NSC extraction with 30 ml of distilled water at 120° for 60 min. The gelatinization was repeated twice.

The abdominal skin collagen of the rats which had the granuloma was also fractionated into NSC and IC by the procedure described above.

An aliquot of each fraction (NSC and IC) was hydrolyzed with 6 N HCl at 105° for 16 hr. Total hydroxyproline in the hydrolysate was measured by the method of Kivirikko *et al.* [9].

*The abbreviations used are: BAPNf, β-aminopropionitrile fumarate; NSC, neutral salt-soluble collagen; GSC, guanidine soluble collagen; IC, insoluble collagen.

CM-Cellulose chromatography. A granuloma pouch was induced in male Sprague Dawley rats weighing 130–150 g by the method already described. D-Penicillamine and BAPNf treatments were similar except that D-penicillamine (40 mg/100 g body wt, equivalent to approximately 80 mg per rat) together with pyridoxine HCl (1 mg/100 g body wt) was injected daily into the granuloma pouch for 8 days.

NSC was extracted by a method similar to that described above and then IC was further fractionated by using 5 M guanidine. Briefly, the residue remaining after NSC extraction was suspended in 30 ml of 5 M guanidine HCl (pH 7.5). Guanidine extraction was carried out by shaking for 4 days at 4° followed by centrifugation. The extraction was repeated twice. NSC and guanidine-soluble collagen (GSC) were obtained as precipitates after the extracts were dialysed against running tap water (16–18°) overnight and then against distilled water at 4°.

An aliquot of the precipitate (NSC or GSC) was dissolved in 10 ml of 0.1 M acetic acid and then dialysed against 0.06 M sodium acetate (pH 4.8). The dialysate was heated at 45° for 30 min and centrifuged at 20,000 *g* for 5 min. The supernatant was chromatographed on a column (16 × 110 mm) of CM-cellulose (Whatman CM-52) at 40° in 0.06 M sodium acetate (pH 4.8). Elution was achieved with a linear gradient of NaCl from 0 to 0.1 M over a total volume of 400 ml at a flow rate of 60 ml/hr. Absorbance of each fraction (4 ml) was measured at 230 nm.

Disc electrophoresis. Samples were dialysed against 0.01 M NaH_2PO_4 (pH 7.2) containing 1 M urea and denatured at 50° for 30 min. Electrophoresis of the denatured samples in sodium dodecyl sulfate acrylamide was performed as described by Furthmayr and Timple [10] in 5% gels for 5 hr at 5 mA per tube. Gels were stained for 60 min in Coomassie Brilliant Blue and destained in 7% acetic acid.

RESULTS AND DISCUSSION

Enhancement of collagenolytic activity by BAPNf. Collagenolytic activity of granulation tissue was determined by measuring the amount of dialysable hydroxyproline formed during incubation *in vitro* of the minced granulation tissue, since it was demonstrated in a previous paper [7] that this reflects the degradation of tissue collagen *in vivo*. As shown in Table 1, BAPNf increased the amounts of free and dialysable total hydroxyproline, suggesting that BAPNf enhanced collagen breakdown of granulation tissue *in vivo*. In accordance with these results, Martin *et al.* [11] found increased urinary excretion of hydroxyproline in BAPN-treated rats.

The solubility of collagen in granulation tissue was markedly increased by BAPNf treatment (Table 1). The CM-cellulose chromatographic pattern of the NSC (Fig. 1) showed that the collagen extracted with 1 M NaCl consisted almost exclusively of γ components, with a small amount of β_{12} component, suggesting that BAPNf inhibited the formation of cross-links of collagen in granulation tissue.

Effect of D-penicillamine on granulation tissue collagen. As shown in Table 2, the collagenolytic activity of granulation tissue was not affected by D-penicillamine treatment. The increase in the solubility of granulation tissue collagen was relatively slight (Table 2) and γ components of the NSC were also slightly increased (Fig. 1), suggesting that the cross-link formation was slightly inhibited by D-penicillamine. On the other hand, D-penicillamine markedly increased the solubility of skin collagen (Table 2). Bailey *et al.* [12] demonstrated that granulation tissue collagen possesses a more stable aldimine cross-link (dihydroxylysinonorleucine [13]) as a major cross-link after reduction) which is not present to any significant

Table 1. Effect of β-aminopropionitrile fumarate on the collagenolytic activity and the solubility of collagen of granulation tissue in rats

	Control	BAPNf-treated
No. of rats		
Net body wt (g)	130.0 ± 2.4	117.1 ± 3.7
Granulation tissue, wet wt (g)	5.22 ± 0.42	4.14 ± 0.24
Collagenolytic activity:		
Free hydroxyproline formed (μg)	22.52 ± 1.33	32.37 ± 2.27†
Dialysable total hydroxyproline formed (μg)	31.38 ± 1.22	43.86 ± 3.86†
Collagen content (mg hydroxyproline):		
Total collagen	10.087 ± 0.923	8.305 ± 1.019
NSC	1.219 ± 0.046	3.461 ± 0.349†
IC	8.868 ± 0.806	4.845 ± 0.738†
NSC/IC × 100 (%)	13.75 ± 1.53	61.44 ± 8.143

Results are means ± S.E.M.

Values significantly different from controls: *$P < 0.05$, †$P < 0.01$.

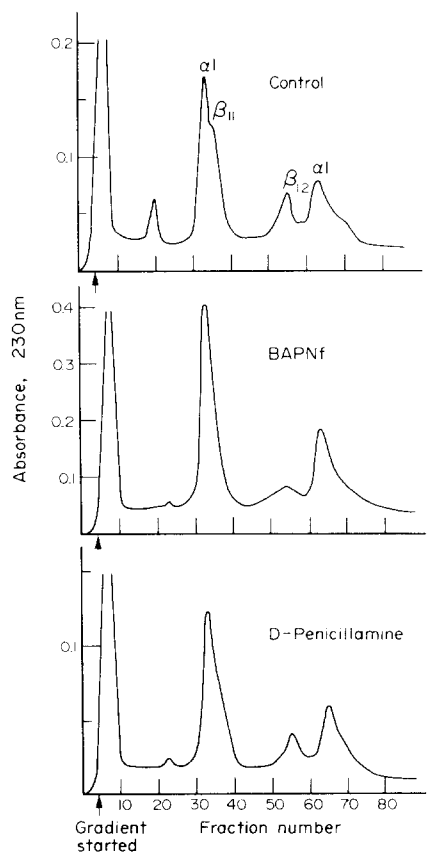

Fig. 1. CM-Cellulose chromatograms of NSC from granulation tissues of control, BAPNf- and D-penicillamine-treated rats. The conditions of chromatography are described in the text.

Table 2. The collagenolytic activity of granulation tissue and the solubility of skin collagen and granulation tissue collagen from rats treated with D-penicillamine

	Control	D-Penicillamine treatment
No. of rats	7	7
Net body wt (g)	142.0 ± 5.2	131.9 ± 3.2
Granulation tissue, wet wt (g)	4.21 ± 0.32	3.25 ± 0.57
Collagenolytic activity of granulation tissue:		
Free hydroxyproline formed (μg)	17.57 ± 1.14	17.08 ± 0.70
Dialysable total hydroxyproline formed (μg)	25.06 ± 2.14	24.20 ± 1.66
Collagen content of granulation tissue (mg hydroxyproline in entire tissue):		
Total collagen	5.130 ± 0.370	4.174 ± 0.910
NSC	1.249 ± 0.093	1.229 ± 0.232
IC	3.881 ± 0.305	2.945 ± 0.685
NSC/IC × 100(%)	34.1 ± 2.0	43.4 ± 3.0*
Collagen content of skin (mg hydroxyproline g wet wt of skin):		
Total collagen	10.586 ± 0.738	8.724 ± 0.483
NSC	4.785 ± 0.306	5.118 ± 0.476
IC	5.801 ± 0.456	3.605 ± 0.157†
NSC/IC × 100(%)	83.6 ± 3.4	144.0 ± 15.0†

The results are means ± S.E.M.

Values significantly different from control: *P < 0.05, †P < 0.01.

extent in normal subcutaneous skin collagen. It is proposed by Deshmukh and Nimni [14] that the cross-linking inhibition caused by D-penicillamine involves a reversible interaction with the aldehydes present in α chains of tropocollagen to form a thiazolidine type complex, while BAPN has a capacity to inhibit the activity of an amine oxidase which converts lysyl residues of α chains into corresponding δ-semialdehydes, aldehydic cross-link precursors [15]. These results suggest that the differences in the solubility of skin and granulation tissue collagen from rats treated with BAPNf or D-penicillamine are based on the differences in the nature of collagen cross-links in these tissues [16] and in the mechanisms of action of both the drugs.

CM-Cellulose chromatography of guanidine extracted insoluble collagen. Approximately 20% of IC was solubilized by 5 M guanidine extraction. After dialysis of the GSC fraction against running tap water (16–18°) and then against cold distilled water (4°), the resultant precipitate was chromatographed on a CM-cellulose column. As shown in Fig. 2, the CM-cellulose chromatographic pattern of GSC was clearly different from that of NSC (Fig. 1). No differences were observed in CM-cellulose chromatographic patterns of GSC from control, BAPNf- and D-penicillamine-treated rats. Each peak labeled I–VI in Fig. 2 was examined by electrophoresis in sodium dodecyl sulfate acrylamide in order to clarify the nature of the peaks. The results are shown in Fig. 3 and 4. Peaks I and II were mixtures of degradation products of α chains. Peaks III, V and VI corresponded to α1, β_{12} and α2 components, respectively. This result was supported by the elution pattern of CM-cellulose chromatography of purified rat tail tendon collagen (Fig. 2). There were no differences in the peaks from control, BAPNf- and D-penicillamine-treated rats. However, peak IV of each group gave a characteristic

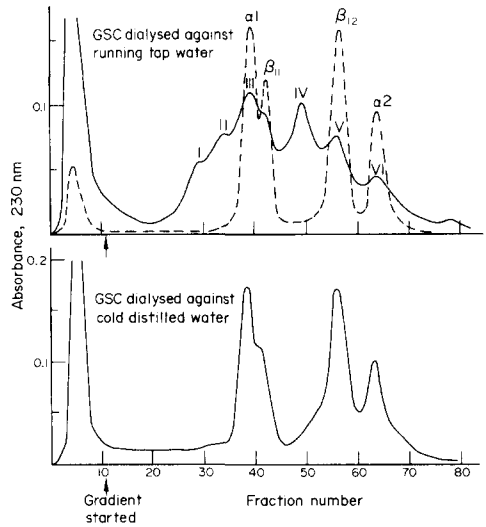

Fig. 2. CM-Cellulose chromatograms of GSC from granulation tissue of control rats. The GSC was obtained by dialysis of GSC fraction against (top) running tap water (16–18°) overnight or (bottom) cold distilled water (1°). Purified rat tail tendon collagen was chromatographed as a standard sample. ——, GSC; – – – –, purified rat tail tendon collagen.

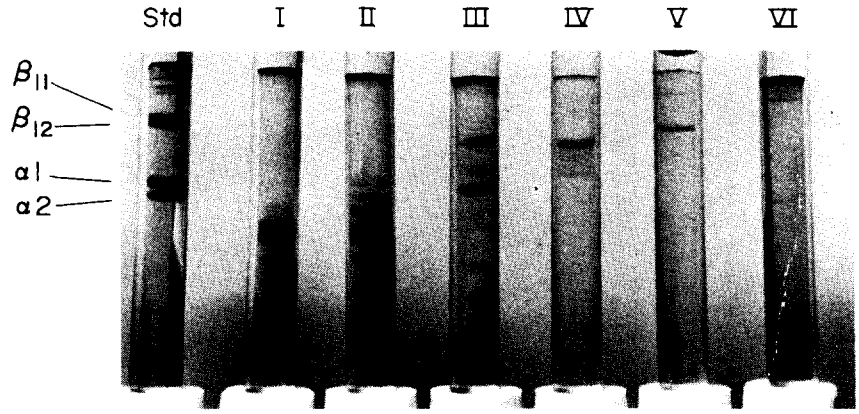

Fig. 3. Sodium dodecyl sulfate acrylamide gel electrophoresis of the peaks isolated by CM-cellulose chromatography of GSC from granulation tissue of control rats. Peaks I–VI labeled in Fig. 2 were examined by disc electrophoresis. The standard (purified rat tail tendon collagen) is on the left.

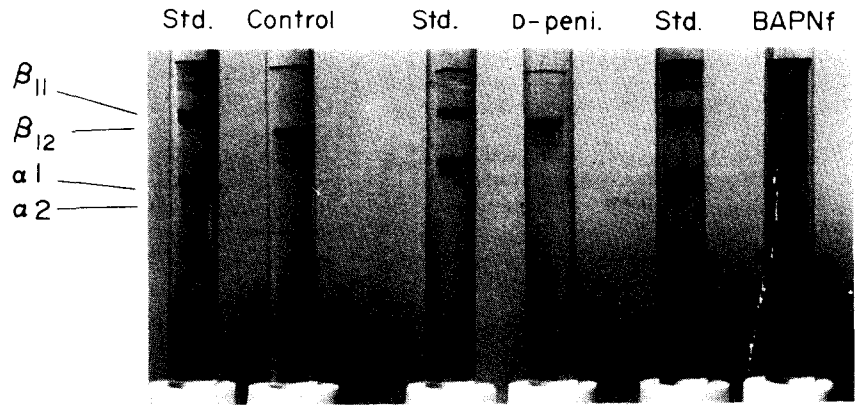

Fig. 4. Sodium dodecyl sulfate acrylamide gel electrophoresis of peak IV isolated by CM-cellulose chromatography of GSC from granulation tissues of control, BAPNf- and D-penicillamine-treated rats. The standard (purified rat tail tendon collagen) is on the left.

pattern upon electrophoresis in 5% sodium dodecyl acrylamide gels. As shown in Fig. 4, peak IV from control and D-penicillamine-treated rats gave a similar pattern on disc electrophoresis. The components of peak IV migrated a little faster than β components, suggesting that the molecular weights were lower than those of β components. Peak IV from BAPNf-treated rats (Fig. 4) had an additional more rapidly migrating component as a main band. The components presented in peak IV may be formed by limited proteolysis of β or γ components. The finding that peak IV from BAPNf-treated group had a lower molecular weight component than those from the control and D-penicillamine-treated groups may reflect the inhibition of cross-link formation and the enhanced degradation of the tissue collagen by BAPNf treatment.

In order to clarify a possibility that a limited proteolysis of GSC may have occurred during dialysis against running tap water (16–18 h), part of the GSC fraction was dialysed against cold distilled water at 1°. As shown in Fig. 2, the CM-cellulose chromatographic pattern of the GSC dialysed against cold distilled water was similar to that of purified rat tail tendon collagen and peaks I, II and IV were not found. It can be presumed therefore, that some proteolytic enzymes are present in the GSC fraction and a limited proteolysis of collagen may have occurred during dialysis of GSC against running tap water.

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